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Inherited DNA Mutations Contributing to Thrombotic Complications in Patients With Sickle Cell Disease

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Thrombosis may play an important role in the pathophysiology of certain complications of sickle cell disease (SCD), including stroke and avascular necrosis (AVN). Currently there is no laboratory or clinical parameter that can identify patients who are at highest risk of developing these thrombotic complications. We hypothesized that some patients with SCD have an inherited hypercoagulable state that results in an increased risk of developing stroke or AVN. We examined the role of two common inherited thrombophilic mutations that, in other populations, have been associated with arterial and venous thrombosis and are amenable to screening with DNA restriction enzyme analysis. The C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene and the C1565T mutation in the platelet glycoprotein IIIa (GPIIIa) gene were evaluated. We analyzed genomic DNA from 86 children and adults with SCD, including 16 patients with a history of a clinical stroke and 14 patients with AVN, for the presence of these mutations. The C677T MTHFR mutation was found in 19% of patients with stroke, 14% of patients with AVN, and 14% of patients with neither complication (*P* = NS). The C1565T GPIIIa mutation was found in 25% of patients with stroke, 14% of patients with AVN, and 18% of patients with neither complication (*P* = NS). Although each of these mutations is relatively common in patients with SCD, neither is independently associated with an increased risk of developing stroke or AVN. Am. J. Hematol. 59:267–272, 1998.

Key words: sickle cell disease; thrombosis; stroke; avascular necrosis; thrombophilic mutations

INTRODUCTION

Sickle cell disease (SCD) is one of the most common inherited diseases in the United States, affecting one in 375 African American live births. SCD is characterized by a predominance of sickle hemoglobin (β^s), which results from an inherited substitution mutation of valine for glutamic acid at the sixth position of beta globin. Patients can be homozygous for this mutation (HbSS) or have β^s in combination with β-thalassemia or another abnormal beta globin chain such as β^c. Patients with SCD have a variety of clinical manifestations including hemolytic anemia, increased susceptibility to infection and other acute and chronic complications that can lead to significant morbidity and mortality [1].

Even with the same inherited DNA mutation within the beta globin gene, patients can have considerable variation in the clinical expression of their disease. Additional genetic factors such as alpha thalassemia [2], fetal hemoglobin (Hb) synthesis [3,4], and beta globin haplotype [5] have been studied, but none of these can fully explain the differences in clinical expression observed in patients with SCD. Predicting which children are at highest risk of developing complications of SCD would provide the opportunity for preventive strategies and therapeutic intervention.

Recently, the importance of thrombosis in the pathophysiology of SCD has been recognized. Complex interactions within the blood vessel resulting from erythrocyte flow disturbances, abnormal erythrocyte adhesion, platelet activation, and alterations in endothelial cell function can lead to thrombus formation [6]. The com-

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bination of intravascular coagulation and ongoing erythrocyte sickling leads to more severe tissue ischemia and organ damage. Clinical complications of SCD that likely have a thrombotic component include vaso-occlusive painful events, cerebrovascular accidents (CVA or stroke), avascular necrosis, priapism, retinopathy, and pulmonary infarction, as well as others [6]. Stroke in children with SCD involves vascular intimal hyperplasia with superimposed thrombosis of large cerebral arteries, perhaps originating at sites of endothelial cell damage [6–8]. Similarly, autopsy reports confirm extensive thrombosis of medium-to-small pulmonary arteries in patients with sickle cell lung disease [6]. Osteonecrosis of bone typically occurs in areas where there is limited collateral circulation and may also involve intravascular thrombosis and hypofibrinolysis [9–13]. Avascular necrosis (AVN) of the femoral heads in other patient populations has been associated with low levels of anticoagulants protein C and protein S, as well as abnormalities of the fibrinolytic system [9–13].

We hypothesized that a subset of patients with SCD have an additional inherited hypercoagulable state that results in an increased risk of developing thrombotic complications. To test this hypothesis, we analyzed the DNA of children and adults with SCD for the presence of two genetic point mutations that lead to an increased risk of thrombosis in other patient populations, including the C677T mutation in the methylenetetrahydrofolate reductase (MTHFR) gene [14–17] and the C1565T mutation in the platelet glycoprotein IIIa (GPIIIa) gene [18]. Both point mutations create a new restriction enzyme cleavage site and are therefore amenable to rapid DNA screening. We evaluated the prevalence of these mutations in patients with SCD and a history of stroke or AVN, two thrombotic complications of SCD with clearly defined diagnostic criteria.

METHODS

Patient Samples

Children and adults with SCD, including those with HbSS as well as HbSC or HbSβ^0-thalassemia, were studied. Relevant clinical information was recorded, including a history of clinical stroke or X-ray documented AVN of the femoral or humeral heads. After informed consent was obtained, approximately 3 ml of whole blood was collected in ethylenediaminetetraacetic acid (EDTA) and stored at room temperature for up to 24 hr. In all cases, the sample was collected at the same time as other blood samples obtained for patient care.

DNA Purification

Genomic DNA purification was performed according to the manufacturer's recommended protocol using a Puregene (Gentra Systems, Inc., Minneapolis, MN) DNA isolation kit. The concentration of DNA was determined by optical density at 260 nm. Samples were stored at 4°C until analyzed.

Polymerase Chain Reaction

Oligonucleotide primers were synthesized by the core facility at Duke University Medical Center. For amplification of the MTHFR gene, the forward primer 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ and the reverse primer 5′-AGGACGGTGCGGTGAGAGTG-3′ were used [17]. For the platelet GPIIIa gene the forward primer 5′-TTCTGATTGCTGGACTTCTCTT-3′ and the reverse primer 5′-TCTCTCCCCATGGCAAAGAGT-3′ were used [18]. The polymerase chain reaction (PCR) was performed using a Perkin-Elmer 4800 thermal cycler (Perkin Elmer, Foster City, CA). Each 100 μL PCR contained: 0.2 μg of genomic DNA template; 2 μL of each PCR primer; 5 units of *Taq* polymerase (Gibco, Grand Island, NY); 20 nmol of each dNTP (Pharmacia Biotech, Piscataway, NJ); 10 μL of 10× PCR buffer (Gibco); 150 nmol of $MgCl_2$; and distilled H_2O. The PCR was initially heated to 94°C for three min for DNA denaturation followed by 40 cycles of PCR amplification. For the MTHFR gene, each cycle consisted of 94°C for 45 sec, 70°C for 90 sec, and 72°C for 60 sec [17]. For the GPIIIa gene, cycles were 94°C for 45 sec, 60°C for 30 sec, and 72°C for 60 sec [18]. PCR products and a 100 bp standard were resolved on a 2.5% agarose gel stained with ethidium bromide and exposed to UV light to document the presence of appropriate sized amplicons.

Restriction Enzyme Digestion

PCR amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA) to remove excess primers, nucleotides, polymerases, and salts. Aliquots of the purified PCR products were run on a 2.5% agarose gel stained with ethidum bromide to document recovery of the expected product. Purified PCR products were then digested with the appropriate restriction enzymes in a total volume of 30–50 μL. Each restriction digestion reaction included 250–500 ng amplified DNA, 1 μL enzyme, 2–4 μL 10× NE Buffer and H_2O. The MTHFR gene product was digested with *Hin*f I (New England Biolabs, Beverly, MA) and the platelet glycoprotein GPIIIa gene product with *Msp* I (Pharmacia Biotech). Reaction mixtures were incubated at 37°C for 2 to 16 hr. Following restriction digestion, samples were run on an agarose gel stained with ethidium bromide and exposed to UV light for photography with a UV filter. Table I summarizes the expected product sizes for each restriction enzyme digestion. Based on the pattern of products, the genotype was determined.

Statistics

Statistical tests were performed using the Primer of Biostatistics (McGraw-Hill, New York, NY) software

TABLE I. Patterns of Expected Product Sizes for Each Restriction Digest*

Gene	Uncut product	Wild type +/+	Heterozygote +/–	Homozygote –/–	Reference
MTHFR	198	198	198		17
			175	175	
			23	23	
GPIIIa	266	221	221		18,39
			177	177	
			50	50	
		45	45	45	

*MTHFR, methylenetetrahydrofolate reductase; GPIIIa, glycoprotein IIIa; PCR, polymerase chain reaction. Genes were amplified by PCR and digested with the appropriate restriction enzymes as described in Methods.

package. Chi-square analysis of contingency tables was used to compare values among groups. The student's paired *t*-test was used to compare mean values between groups.

RESULTS

Clinical Characteristics

We studied 86 patients with SCD including 76 with HbSS, nine with HbSC, and one with HbSβ^0-thalassemia. There were 32 females and 54 males. The patients ranged in age from five to 60 years with a mean age of 18 years and a median age of 15 years. The younger patients were those with a history of clinical stroke. Sixteen patients (20%) had a prior stroke and 14 patients (16%) had X-ray proven AVN of the femoral or humeral heads. One patient had both complications. All of the patients with stroke had HbSS while two of the 14 patients with AVN had HbSC disease. Of the 57 patients with neither complication, seven had HbSC and one had HbSβ^0-thalassemia.

Analysis of the C677T MTHFR Mutation

An example of the restriction enzyme digestion pattern for the C677T MTHFR mutation is shown in Figure 1. No homozygotes were identified, but 13 of the 86 patients were heterozygous for the mutation for an overall prevalence of 15%. Heterozygotes for the C677T MTHFR mutation included three of the 16 patients (19%) with stroke, two of the 14 patients (14%) with AVN, and eight of 57 patients (14%) with neither complication. These differences were not statistically significant.

Analysis of the C1565T Platelet Glycoprotein IIIa Mutation

An example of the restriction enzyme digestion pattern for the C1565T GPIIIa mutation is shown in Figure 2. No homozygotes were found, but 16 of 85 patients were heterozygous for the mutation for an overall prevalence of 19%. In one patient, no result was obtained because of an insufficient DNA sample. The heterozygous C1565T GPIIIa mutation was found in four of the 16 patients (25%) with stroke, two of 14 patients (14%) with AVN, and 10 of 56 patients (18%) with neither complication. These differences were not statistically significant. Three patients were heterozygous for both the C677T and the C1565T mutations, one of which had a history of AVN.

DISCUSSION

There are several pieces of evidence that indicate that activation of the coagulation system occurs in patients with SCD, including platelet activation [19,20] and thrombin generation [21,22]. Fibrin D-dimer levels increase in SCD during acute painful events and are significantly elevated in most patients with leg ulcers, aseptic necrosis, and stroke [21,23]. Increased plasma fibrinogen levels may contribute to localized vasoocclusion by effects on platelet and red cell adhesion and blood viscosity [24]. Circulating anticoagulant factors such as protein C, protein S, and antithrombin III are typically low in patients with SCD [25]. Taken together, these findings suggest that activation of coagulation and resulting thrombosis may contribute to the clinical complications of SCD. There is currently no single clinical or laboratory parameter, however, that can predict which children are at highest risk for developing thrombotic complications of SCD. Some patients with SCD could have an additional inherited predisposition to thrombosis that, in combination with other endothelial, erythrocyte, and coagulation abnormalities, leads to an increased risk of thrombotic complications such as stroke or AVN. We therefore examined the role of inherited mutations in the MTHFR and platelet GPIIIa genes that have been associated with an increased risk of thrombosis in other patient populations [14–18].

An elevated plasma homocysteine level is known to be an independent risk factor for thrombosis, including cerebral, peripheral, and coronary vascular disease [16,26–31]. Hyperhomocysteinemia can result from a variety of genetic enzymatic defects within the biochemical pathway of folate metabolism, including rare homozygous deficiencies of cystathione β-synthase or MTHFR. The

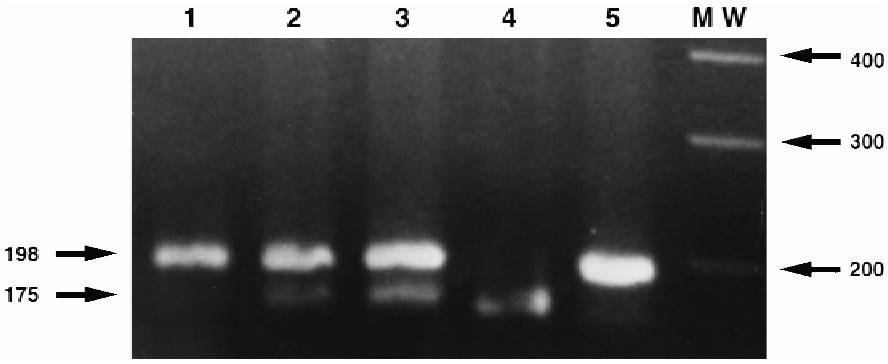

Fig. 1. Restriction enzyme digestion of the C677T MTHFR mutation. Molecular weight markers (100 bp ladder) are shown on the right. The 23 base pair (bp) product is not shown on the gel. Lane 1 shows a digested sample from a patient homozygous for the normal allele. All of the product is 198 bp. Lanes 2 and 3 are from patients that are heterozygous for the normal allele and the C677T mutation, and show products at both 198 and 175 bp. Lane 4 is a sample from a patient who is homozygous for the C677T MTHFR mutation with all of the digested product at 175 bp. Lane 5 shows an undigested sample.

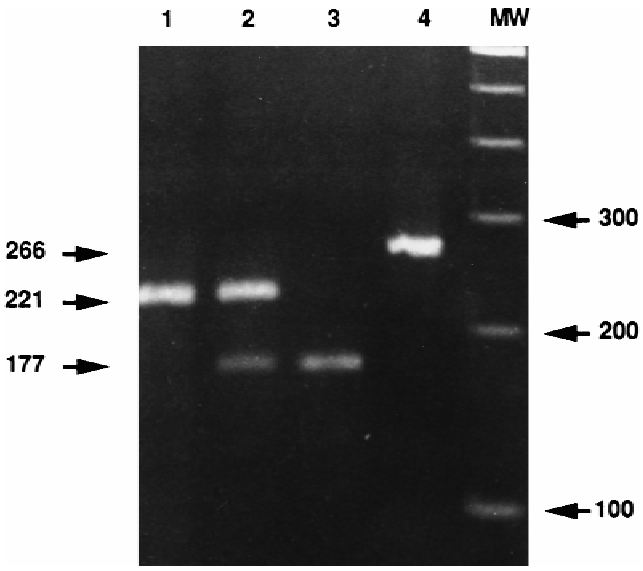

Fig. 2. Restriction enzyme digestion of the C1565T GPIIIa mutation. Molecular weight markers (100 bp ladder) are shown on the right. The 45 and 50 bp products are not shown on the gel. Lane 1 shows a digested sample from a patient homozygous for the normal allele with all of the product at 221 bp. Lane 2 is from a patient heterozygous for the normal and the C1565T mutation and products are seen at 221 bp and 177 bp. Lane 3 is a sample from a patient who is homozygous for the C1565T GPIIIa mutation. All of the digested product is 177 bp. Lane 4 is an undigested sample of 266 bp.

pathophysiology of this thrombophilic state is not well understood, but homocysteine may damage vascular endothelium, thus providing a thrombogenic nidus in the vessels, or stimulate proliferation of smooth-muscle cells, a key component in atherogenesis [27]. A common cause of hyperhomocysteinemia, however, is a thermolabile variant of MTHFR associated with partial enzyme activity and elevated homocysteine levels [32,33]. A common mutation in the MTHFR gene, a substitution of C→T at nucleotide position 677 that changes alanine to valine, correlates with reduce enzyme activity and thermolability. This mutation creates a new restriction site that can be detected by restriction enzyme analysis. Whereas all patients with the C677T MTHFR mutation have significant enzyme thermostability, only homozygotes have significantly elevated plasma homocysteine levels [17]. This mutation is emerging as an important inherited risk factor for thrombotic disease [17,34–37]. It occurs in up to 38% of an unselected French-Canadian population [17], but its prevalence in other ethnic groups may vary [34–37]. A recent report found the incidence of homozygosity in African Americans to be 1.4% [38]. We found that the C677T MTHFR mutation was present in 15% of our patients, similar to findings recently reported in abstract form [38]. Patients with stroke or AVN had a similar mutation prevalence, however, indicating that the C677T MTHFR mutation is not an independent risk factor for the development of these complications.

Inherited abnormalities of platelet surface receptors have also been investigated as independent risk factors for thromboembolic disease. A genetic polymorphism of platelet glycoprotein IIIa, a membrane receptor for fibrinogen with a central role in platelet aggregation, has been recently identified [18,39–41]. This Pl^{A2} polymorphism is characterized by a C→T at nucleotide 1565 in exon 2 and leads to a change of leucine to proline. The mutation creates new restriction sites so that the mutation can be identified by restriction enzyme analysis [18,39,41]. The C1565T GPIIIa polymorphism has been strongly associated with acute coronary thrombosis, especially in patients with early onset of their disease [39,41]. The prevalence of the C1565T mutation ranges from 15–26% and may also vary in different ethnic groups [39–41]. We found that the C1565T GPIIIa mutation was present in 19% of patients with SCD. Patients with stroke or AVN had a similar mutation prevalence,

however, indicating that the CT1565T GPIIIa mutation is not an independent risk factor for the development of these complications.

Although the C677T MTHFR and C1565T GPIIIa mutations were not over-represented in patients with stroke or AVN, some of our younger patients may develop these complications over time. It is still possible that other inherited thrombophilic mutations contribute to thrombotic complications in SCD. The Factor V Leiden (G1691A) mutation that results in activated protein C resistance is not associated with the development of stroke in patients with SCD [42], but other mutations have not been studied. Mutations and polymorphisms in the fibrinogen [43–50], prothrombin [51], and Factor VII [52] genes should be analyzed to determine the contribution of inherited thrombophilic mutations to thrombotic complications in patients with SCD.

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